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(71) Applicant (for all designated States except US):
FORSKARPATENT I LINKÖPING AB [SE/SE]; S-
581 83 Linköping (SE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): LUNDSTRÖM, Ingemar
[SE/SE]; Färgaregatan 10, S-582 52 Linköping (SE).
TENGVALL, Pentti [SE/SE]; Blåklintsgatan 4, S-582
46 Linköping (SE). LESTELIUS, Magnus [SE/SE];
Mårdtorpsgatan 53, S-582 48 Linköping (SE). NYGREN,
Håkan [SE/SE]; Moss Gård, S-427 38 Billdal (SE).

(74) Agent: BERGLUND, Erik; Forskarpatent i Linköping AB, S-
581 83 Linköping (SE).

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(54) Title: GLUTATHIONE MODIFIED SURFACES

(57) Abstract

The invention relates to a novel thiol modified substrate or article with a noble metal or polymer surface on to which glutathione has been immobilized, said novel product possessing an outstanding combination of biological activities making it possible for e.g., blood contact uses, such as an implant. The product is preferably prepared by chemisorption of the glutathione from an aqueous solution thereof.

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GLUTATHIONE MODIFIED SURFACES

Technical field

The present invention is within the field of substrates having at least a surface layer modified by an immobilized thiol compound. More specifically it relates to the immobilization of surface layers by a specific novel thiol compound which has unexpectedly been shown to possess outstanding biological activities as compared to previously known immobilized thiol compounds. The invention also relates to some specific favourable uses of said substrate, said uses being made possible by the new activities found.

Background of the invention

Gold surfaces modified by immobilized thiol compounds are previously known per se. Generally such modified surfaces have appeared to be of interest as model systems for the study of protein-surface interactions. It has also been found by the inventors that the thiol-gold immobilization technique is a reliable technique for studies of coagulation and complement activation under well controlled conditions. Thus, the surface modification technique offers possibilities to separate the influence of different functionalities from those of physical factors, such as surface mobility (tail group) and morphology (surface roughness).

As an example of prior art within this field reference can be made to P. Tangvall, M. Lestelius, I. Lundström and B. Liedberg "Plasma Protein and Antisera Interactions with L-cysteine and 3-Mercaptopropionic Acid Monolayers on Gold Surfaces", *Langmuir*, 8, 1236-1238 (1992). In this recent study it was shown that short and fairly simple thiols, like MPA ($\text{HSCH}_2\text{CH}_2\text{COOH}$) and L-cys ($\text{HSCH}_2\text{CH}(\text{NH}_3^+)\text{CO}_2^-$), immobilized onto gold, bound different antibodies after incubation in human plasma. Both surfaces are hydrophilic but with different net charges and chemical compositions.

Polymer surfaces modified with immobilized molecules are of course also previously known.

General description of the invention

5 According to the present invention it has unexpectedly appeared that a specific thiol that is more complex as to chemical structure than those fairly simple thiols which have hitherto been investigated for protein-surface interactions, viz. glutathione, shows outstanding properties when immobilized onto a noble metal surface. More specifically it has been found that said immobilized molecule is essentially completely inactive both with reference to coagulation activity and with reference to complement activity. Thus, although some molecules have previously been found to possess a low coagulation activity, they have in fact not been useful for medical purposes in practice due to a pronounced complement activity, i.e. they activate the complement system of the body and provoke the cell injurious effects thereof. That is, generally they cause inflammatory reactions.

20 In other words the new immobilized system according to the present invention shows a unique combination of non-adverse activities which makes it possible for a large number of medical applications, especially where blood contact is involved, particularly for short time blood contacts, such as up to about 2 hours. For instance this means that the new system should be a competitor to the well known technique of heparinizing surfaces to be used for different medical purposes. The fact that it may well be a strong competitor to heparin, especially at short time contacts, is not only because of its outstanding biological activities but also a result of the fact that the method of immobilizing glutathione onto a noble metal surface is extremely simple and reliable, as will be discussed more below.

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Thus, a first object of the invention is to provide a new modified substrate possessing a unique combination of biological activities which makes it possible for a large number of valuable medical applications.

5 Another object of the invention is to provide a modified substrate that can be manufactured or prepared in an extremely simple and reliable way.

Still another object of the invention is to provide a modified substrate carrying an immobilized thiol compound
10 that is non-synthetical, i.e. completely native to the human or animal body, as said substance per se is a substance found in blood and human and animal tissues.

One additional object of the invention is to provide a modified substrate, the other part of which, i.e. in addition to the thiol ingredient, is completely inactive or
15 inert to its environment as it is of a noble metal.

Still another object of the invention is to provide a very simple and reliable process for the preparation of the above-mentioned substrate.

20 Other objects of the invention include the use of the modified substrate referred to in various medical articles and assays for different medical or diagnostic purposes.

Furthermore, the immobilized new thiol compound should work similarly also on a polymer surface, which
25 means that one additional object of the invention is to provide a modified substrate having at least a surface layer of a polymer, to which said thiol has been immobilized. Such a polymer article should be especially well suited as a disposable article.

30 Other objects of the invention should be obvious to a person skilled in the art when reading the present description or claims.

More specifically, according to a first aspect of the invention, there is provided a substrate of the type having at least a surface layer modified by an immobilized
35 thiol compound, the characteristic feature of said substrate being that said surface layer is a noble metal

or polymer layer modified by immobilized glutathione.

In connection with the invention the term noble metal is utilized in its common sense, i.e. generally a metal or alloy that is relatively superior in resistance to corrosion or oxidation. Generally it can also be said that the
5 term is an equivalent to precious metal.

As such noble metals have previously been utilized to immobilize thiol compounds reference can also be made to the prior art concerning details of said metals. Preferably, however, the noble metal is selected from the group
10 consisting of gold, silver and platinum, most preferably gold.

The term polymer generally relates to rather inert polymers, especially such which have been modified according to previously known techniques. Preferably said polymer is selected from the group consisting of polyethylene, polypropylene, polystyrene, polyurethane or terephthalates.
15

The term substrate is used in a broad sense in connection with the invention. Thus, generally it means any
20 part, substance, element, etc., which lies beneath and supports another, i.e. in this case it supports the glutathione. In other words the substrate may well be a part of a larger article, e.g. a medical article, or represent such an article per se. A person skilled in the art should
25 have no problem in making a proper choice of said substrate, neither as to configuration or shape, nor as to the nature thereof. In general the nature of the substrate is selected according to known principles, as this part of the invention also represents prior knowledge. Preferably,
30 however, the substrate base material is selected from the group consisting of silicon, glass, polymer and metal. Theoretically the substrate might be of said noble metal in its entirety, but for obvious cost reasons this is not the case for larger articles.

35 On the contrary integral articles made completely from the polymer referred to are possible for cost reasons.

Furthermore, it has been found that the invention works extremely well with a rather thin surface layer of said noble metal or polymer, preferably within the range of 50-500 nm. According to an especially preferable embodiment of the invention the thickness of said surface layer is 100-300 nm, such as approximately 200 nm.

As concerns the technique of accomplishing the noble metal surface layer a preferable embodiment of the invention means that said noble metal layer has been sputter-deposited or physically vaporized onto the substrate referred to. Such sputtering technique is well known and has been utilized per se in connection with immobilizing thiol compounds onto gold surfaces, and reference is therefore made to prior literature as to the preparation of said noble metal layers.

How to provide a surface layer of the polymer should be well-known to a person skilled in the art and need not be described here.

With reference to the thickness of the immobilized glutathion layer it has been found in accordance with the present invention that a preferable range thereof is 5-25 Å, preferably 5-13 Å, e.g. about 10 Å. Extremely good results have been obtained when using a mono-layer of glutathione immobilized on said surface layer.

According to still another preferable embodiment of the invention the substrate is a substrate for which the glutathione has been immobilized on to the surface layer by chemisorption from an aqueous solution thereof, which technique is especially preferable for said noble metal layer. Preferably the concentration of said aqueous solution of glutathione is within the range of 0.1-10 mM. Otherwise the technique of immobilizing the glutathione molecule is similar to the techniques already used for immobilizing other thiol compounds onto gold surfaces. Therefore, further details concerning this part of the invention as well as the immobilization onto polymer surfaces can be found in relevant literature. In addition

thereto further details about the immobilization will of course be found in the more detailed description of the invention below.

According to a second aspect of the invention there is also provided a process for the preparation of the substrate defined above. Said process, which is very simple and reliable and which may even be used by the final user of the substrate, is characterized by starting from a substrate having a noble metal or polymer surface or alternatively sputter-depositing on a substrate a noble metal surface and modifying said surface with glutathione by chemisorption from an aqueous solution of said glutathione so as to immobilize the same thereupon.

As referred to above the technique of sputter-depositing the noble metal onto a substrate is well known and such principles may be used here. Although further details can be found in the literature it may perhaps be added that to improve the adherence of the noble metal layer to the substrate a very thin layer of an adherence-promoting metal, such as chromium, could be used. Furthermore, the surface is preferably cleaned, for instance in distilled water, ammonium hydroxide and/or hydrogen peroxide, before immobilizing the glutathione. In addition thereto the surface is also rinsed, for instance in distilled water and stored therein until the chemisorption takes place.

The chemisorption of the glutathione can also be performed according to known principles. For instance this means that it may well be performed at room temperature or at slightly elevated temperatures, which is of course extremely advantageous.

After the chemisorption step the glutathione layer is preferably treated by sonication, which sonication is suitably performed after having rinsed the glutathione layer in water, such as distilled water.

According to still another aspect of the invention there is also provided an article which comprises a substrate as defined above or as manufactured in the above-

described way, for use in medical or diagnostic treatment of the human or animal body. In this context the term medical is to be understood in a very broad sense, i.e. the article referred to need not primarily be used for a therapeutic purpose but may well be utilized for applications like implants, surgical instruments and sensors. However, as referred to above, the article is useful for almost any application where coagulation and complement activities are to be avoided, such as in all contacts with blood. For instance this means that the article is well suited for cell culture uses. However, other medical uses should be obvious to a person skilled in the art and need not be enumerated here.

Thus, according to an additional aspect of the invention there is also provided a use of the substrate as defined above or as manufactured above, for the manufacture of any article to be used in blood contacting medical or diagnostic treatments or surgical treatment of the human or animal body. As mentioned above, this may for instance be a use of the substrate for the manufacture of an implant or a surgical instrument article.

Examples

The invention will now be further described more specifically by means of working examples where the immobilized glutathione according to the invention is prepared and compared to the previously known immobilized molecules 3-mercaptopropionic acid (MPA) and L-cysteine (L-cys).

Before presenting the examples, however, the Figures referred to therein should be briefly commented on. Thus, in the accompanying figures the following is shown:

Fig. 1 Suggested molecular structures of 3-mercaptopropionic acid, L-cysteine and glutathione respectively, at physiological pH (7.4)

Fig. 2 FT-IRAS spectra of a) 3-mercaptopropionic acid, b) L-cysteine and c) glutathione monolayers on gold, formed at spontaneous pH (3.3, 5.3 and 3.4, respectively).

5

Fig. 3 Kallikrein formation and indirect quantification of F XII and F XII_a on surfaces and in solutions. The sodium glass surface was used as a highly activating reference.

10

Fig. 4 Anti-C3c depositions (15 min incubations) after incubation in human serum for 10 min, under room conditions.

15 Fig. 5 Total plasma protein and antisera adsorptions onto gold, gold-MPA, gold-L-cys and gold-GSH surfaces at room conditions. The surfaces were incubated in 10% citrated human plasma for 10 min and then in antisera for 15 min (for details, see Materials & Methods). In a) the horizontal dashed line indicates the over-all plasma level and all samples to the right of the vertical dashed line have been treated with amplification antisera. In b) all samples to the right of the dashed line
20 have been treated with amplification antisera.
25

Material and Methods

Gold films, about 200 nm thick each, were sputter-deposited on glass substrates precoated with a thin layer
30 of chromium ~ 1 nm. Scanning force microscopy (contact mode, 400 nm scanside) indicated that the surfaces were flat with a surface roughness ≤ 6 nm (see Table 1). The gold surfaces were cleaned in five parts (v/v) distilled water, one part ammonium hydroxide (25%) and one part of
35 hydrogen peroxide (30%) for 10 min at 80°C. After the cleaning procedure, the hydrophilic surfaces were rinsed and stored in distilled water for no more than 15 min

before chemisorption of the thiol.

Monolayers of glutathione (GSH), L-cysteine (L-cys) and 3-mercaptopropionic acid (MPA) were formed in aqueous solutions of 1 mM L-cys ($\text{pH} = 5.3 \pm 0.3$) (from Sigma), 1 mM MPA ($\text{pH} = 3.3 \pm 0.3$) (from Fluka) for 15 min, or in 2 mM GSH ($\text{pH} = 3.4 \pm 0.1$) (from Sigma) for 1 hour, at room temperature (RT). After chemical modification the surfaces were rinsed in distilled water and finally sonicated for 3 min before contact angle and ellipsometric measurements were made (for surface characterization, see Table 1).

The molecular structure of the formed monolayers were analysed using Fourier Transform Infrared Reflection-Absorption Spectroscopy (FT-IRAS) in a dry state under mild vacuum. The analyses were performed using a BRUKER IFS 113 v Fourier transform spectrometer, equipped with a DTGS detector and a GIR (Grazing angle Infrared Reflection) accessory aligned at 83° . All spectra were obtained at a spectral resolution of 4 cm^{-1} by averaging 500 interferograms.

Prior to plasma incubations, one of six identically prepared surfaces was rinsed in distilled water, blown dry in nitrogen, and the complex refractive index of the surface was calculated from ellipsometric measurements in ten different points.

The sample-surfaces were incubated for 10 min in 10% citrated human plasma in 0.01 M Phosphate Buffered Saline (PBS, 82 volume % 0.01 M Na_2HPO_4 , 18 volume % 0.01 M KH_2PO_4 and pH setting to 7.4 by NaOH/HCl), at RT. After rinsing in buffer they were immersed in antisera at 1/20 or 1/50 dilutions for 15 min. Some samples were then transferred to the amplification antisera (1/50 dilution, 15 min). After rinsing in distilled water and drying in nitrogen the amount of organic material was determined by ellipsometry at five points on each sample. Five samples for each antisera were analysed. The equivalent organic layer thickness was calculated from the ellipsometric data

using an isotropic three-phase model and a refractive index $n = 1.465$ for the organic layer. The maximum error of the calculated organic layer thicknesses was low, ± 0.3 nm. The error of the calculated thickness was determined using the formula for pooled standard deviations, the standard deviation was then multiplied with values from the Student's t-table (95%-level of confidence). The error bars in figures 4-5 thus represent the calculated error (from standard deviation) plus the estimated error for the ellipsometer (± 0.3 nm). The thicknesses of the L-cys, MPA and GSH layers were calculated from the changes in the optical properties of the gold surface after the chemical modification. The ellipsometer used was a Rudolph Research AutoEl III, equipped with a He-Ne laser working at 632.8 nm.

Citrated plasma and serum were prepared from blood from apparently healthy donors and stored at -80°C until use. The IgG fraction antisera used were Swine anti-Albumin (SwaAlb), Swine anti-Immunoglobulin G (SwaIgG) and Swine anti-Complement 3c (SwaC3c) from Orion Diagnostica, Finland; Rabbit anti-Fibrinogen (RbaFG), Rabbit anti-Fibronectin (RbaFN), Rabbit anti- α_2 Macroglobulin (Rba α_2 M) and Rabbit anti-Complement 3c (RbaC3c) from Dakopatts a/s, Denmark; Goat anti-High Molecular Weight Kininogen (GtaHMWK), Goat anti-Factor XII (GtaFXII), Goat anti-Factor VIII (GtaFVIII), Goat anti-AntiThrombin III (GtaATHIII), Goat anti-Lipoprotein (GtaLp) and Goat anti-Prekallikrein (GtaPK) were all from Nordic Immunochemical Laboratories, the Netherlands. Human Serum C-Reactive Protein (CRP) Calibrator and Goat a CRP were from Dakopatts a/s, Denmark. The surface concentrations of low concentration plasma proteins (HMWK, LP, FXII, FVIII, ATH III and PK) were amplified with secondary antibodies: Rabbit anti-Swine Immunoglobulins (RbaSw) and Rabbit anti-Goat Immunoglobulins (RbaGt), both from Dakopatts a/s. Control tests showed only a small crossreactivity with plasma proteins, and the amplification antisera had low

affinity for surfaces incubated in human plasma only. A small amount of human IgG was added to diluted amplification antisera to further suppress the crossreactivity, as recommended by the suppliers. Subsequently, the results should therefore be compared only for each protein and do not allow quantitative comparisons between different proteins on a specific surface.

Complement factor deposition on solid surfaces can be detected using ellipsometry and antisera techniques. Here, detection of complement-activation, as indicated by anti-C3c binding, was performed in the following manner. Surfaces were incubated in 10% serum diluted in HANK's buffer (solution A: 30 g NaCl, 4 g KCl, 2 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.94 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 500 ml distilled water; solution B: 0.6 g Na_2HPO_4 , 0.6 g KH_2PO_4 , 10 g dextrose and 500 ml distilled water, A and B were mixed and the pH set with NaOH/HCl) at pH 7.4 for 10 min, rinsed in buffer followed by distilled water and blown dry. The adsorbed layer thickness was measured in five points. The surfaces were again rinsed in buffer and incubated in an anti-C3c solution diluted 1/50 in HANK's, for ten minutes. Finally, the surfaces were removed and rinsed with buffer and distilled water, blown dry and the organic layer thickness was once more determined in five points. Parallel to this, a control set of surfaces was incubated in heat deactivated 10% serum (56°C for 30 min before use) and antisera as above. The difference in the amount of deposited anti-C3c for the two series was interpreted as a measure of the relative degree of complement activation.

Surface bound kallikrein and kallikrein in solution were determined using soda watch-glass surfaces covered with (200 nm thick) gold films. The gold films were made by thermal evaporation on top of a thin layer (4 nm) of chromium. Pure glass, known to be a rapid contact activator, served as the reference material. The glass, gold and thiolated gold watch-glasses were cleaned like the other gold substrates and incubated in 10% citrated human plasma

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in PBS, pH 7.4, for 10 min. The surfaces were then rinsed 3 times in 0.05 M Tris buffer (25 ml Tris-(hydroxymethyl)-amino-methane, 42 ml 0.1 M HCl and 100 ml distilled water) plus 0.15 M NaCl, at pH 7.5. The kallikrein assays (described below) were made using normal citrated plasma, citrated factor XII deficient (F XII_{def}) plasma from Sigma as the extra prekallikrein source, and Cephotest (cephalin, phosphatidylethanol-amine) from Nycomed AB, Lidingsö, Sweden, was used to activate F XII. The kallikrein-specific H-D-Pro-Phe-Arg-pNA·2HCl peptide S-2302 was from Chromogenix AB, Mölndal, Sweden. The principle of the test is kallikrein cleavage of the S-2302 substrate. The end product (pNA) has a high absorbance at 405 nm and is quantified by spectrophotometry.

15

Peptide-pNA (S-2302) kallikrein → Peptide-OH+pNA (yellow, 405 nm).

The kallikrein assays were performed as follows:

20 The surfaces were rinsed thoroughly with Tris buffer and incubated in 400 µl, 10% (PBS, pH 7.4) plasma for ten min. The rinsed surfaces were then tested for (the boxes indicate additions into the watch glasses after plasma treatment) total amount of surface bound Factor XII.

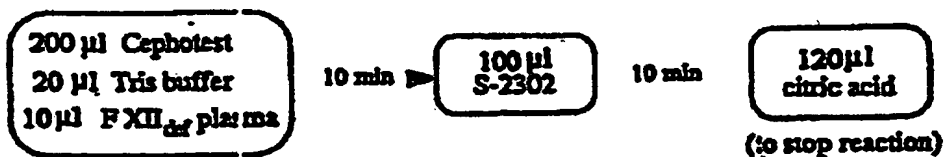
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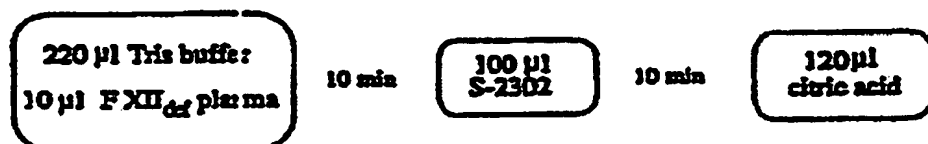
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activated Factor XII (FXII_a) on the surface

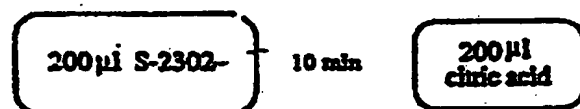
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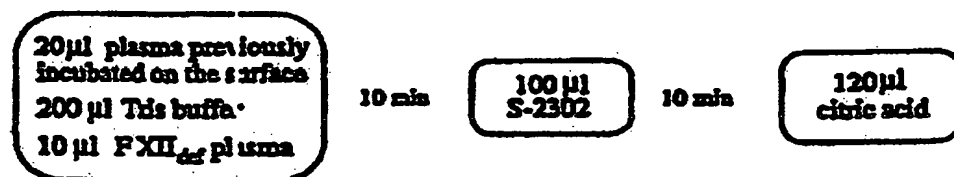
kallikrein on the surface

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The plasma used in the incubations was tested for
factor XII_a

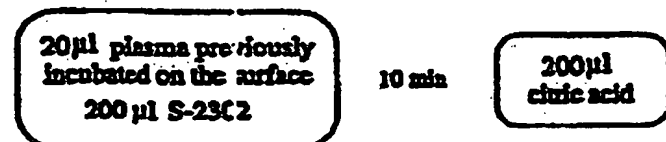
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30

kallikrein

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The assay solutions were transferred into a 1 ml polystyrene disposable cuvette, the volumes were doubled with Tris buffer, and the absorbance was measured at 405 nm. The errors presented in Figure 3 are maximum errors.

5 The number of tests for each component varied from three to six for the various surface modifications.

Results and Discussion

Suggested molecular structures

10 All three modified surfaces were hydrophilic with water contact angles smaller than 15° . The MPA and L-cys are likely to give weakly negatively charged and zwitterionic surfaces, respectively, at physiological pH. Glutathione presents one negatively charged and one zwitterionic end, separated by a polypeptide-like backbone as shown in Figure 1.

15

Surface characterization with FT-IRAS

20 The reflection absorption (R-A) spectra of MPA, L-cys and GSH are shown in Fig. 2. There exist unfortunately no complete assignments of the spectra of these molecules in the literature. However, the peak positions of the strongest bands and their tentative assignments are summarized in Table 2. All three R-A spectra lack the characteristic peak of the SH group at 2550 cm^{-1} (spectral region not shown in Fig. 2), confirming that the binding to the gold surface occurs via the thiol group. The peak positions and general appearance of the R-A spectra for the L-cys and MPA monolayers correspond well with prior art.

25

30 Looking at the spectrum of GSH, the most intensive peaks are those originating from the amide groups. The so-called Amide I peak (C=O stretching) occurs at 1663 cm^{-1} and the Amide II and III peaks (C-N-H stretching and bending) occur at 1549 and 1242 cm^{-1} , respectively. For the carboxyl groups (COOH) of GSH, the C=O stretching mode at 1728 cm^{-1} , reveals the acid form. It should be emphasized, however, that all three monolayers investigated in this

35

study are prepared at a significantly lower pH than employed in the plasma protein adsorption experiments. The occurrence of charged and dipolar species (CO_2 and NH^+) at physiological pH is therefore expected to be more frequent as indicated in Fig. 1.

Kallikrein assays

In normal coagulation activated blood factor XII_a (or the Hageman factor) cleaves prekallikrein to form kallikrein, which in turn activates high molecular weight kininogen (HMWK) and inactivate F XII, thus resulting in the intrinsic coagulation cascade. The relative amounts of total surface bound F XII, surface bound activated F XII (F XII_a), surface bound kallikrein, plasma F XII_a , and plasma kallikrein for the various surfaces are shown in Fig. 3. The glass and MPA surfaces bound comparatively large amounts of F XI and F XII_a and released kallikrein into solution.

L-cys and GSH showed low or undetectable kallikrein formation in all the assays (Fig. 3), indicating a low surface F XII activity. This was expected for L-cys since contact activation of coagulation is known to preferentially take place on negatively charged surfaces and L-cys is suggested to be non-charged (zwitterionic) at pH 7 (Fig. 1). The low kallikrein formation on GSH is, however, not yet understood, since significant amounts of COO-groups are expected to be present at pH 7.

Gold exhibited large and significant amounts of surface bound F XII and kallikrein, respectively, although the plasma levels appeared to be low. The low release of kallikrein from the gold surface indicates a low F XII_a formation in plasma. This in turn suggests that gold binds and activates F XII, but the F XII_a and kallikrein turnover is low. The results may be due to plasma proteins being tightly bound to the surface.

16

Control experiments for unspecific kallikrein formation with pure gold substrates showed that

i) no kallikrein was formed in any assay when using F XII_{def} plasma instead of normal. This shows that F XII
5 was a prerequisite for kallikrein formation

ii) kallikrein was formed by using cephalin in the plasma after incubation on Au, implying that the plasma was not depleted of F XII or kallikrein.

iii) a layer of low but still significant thickness
10 of the S-2302 reagent was adsorbed, on top of the plasma protein layer on gold, as measured by ellipsometry. This demonstrates that the reagent was also surface active.

iv) experiments performed with 1 or 20 min plasma incubations gave similar results.

15 This indicates that the incubation time chosen for the present set of experiments was not the cause for the obtained results.

Another set of control experiments using glass substrates incubated in prekallikrein deficient plasma gave
20 negative results for kallikrein in plasma and positive when tested for surface bound F XII. Thus again, F XII was necessary for the colouring to occur. In summary, the control tests showed that the pNA formation was correlated to both the presence of F XII and prekallikrein on surfaces
25 or in plasma.

Deposition of anti-C3c

Ellipsometry was used to detect the increase in thickness of the organic layer after serum and subsequent
30 anti-C3c incubations. The results are shown in Figure 4. L-cysteine surfaces incubated in normal serum showed a small thickening of the organic layer after incubation in the antibody solution. The results were similar when citrated plasma was used instead of serum.

Antisera binding

In Figures 5a and 5b the amounts (equivalent thicknesses) of deposited organic material on the surfaces after plasma and antisera incubations are summarized. In this context it should be noted that the observed amount of organic material in each case is the amount which remains after the rinsing and drying steps.

Pure gold deposited significant quantities of a-IgG, a-FG, a-ATH III, a-PK and a-HMWK (Fig. 5a). The deposition of a-HMWK and a-PK suggests that the surface may be coagulation active, although no a-F XII bound onto the surface. The kallikrein assays, however, revealed the presence of FXII on the surface (see Fig. 3) and that only low amounts of kallikrein were released into the solution.

The MPA surfaces bound relatively large amounts of a-HMWK (a-amplification antisera) and significant quantities of a-F XII, a-ATH III and a-PK (Fig. 5b). The low overall a-FG binding onto the investigated hydrophilic thiol modified surfaces and the large a-HMWK binding onto the (negatively charged) MPA surface (-COO-), are not surprising. The anti-HMWK followed an expected binding pattern of HMWK, i.e. the histidine rich positively charged portion of HMWK may be deposited onto the negative MPA surface, perhaps physisorbed via electrostatic interaction. The kallikrein assays (Fig. 3) also showed elevated F XII_a and kallikrein formation on and outside the MPA surfaces. The combined antisera binding and kallikrein assay results suggest that MPA is clot promoting, when immobilized onto gold.

The L-cys surfaces bound significant amounts of a-IgG, a-ATH III, a-LP and a-PK and relatively low amounts of a-FG and a-HMWK after the plasma incubations. The significant a-IgG deposition (see Fig. 5b) could be related to the increased a-C3c binding after exposure to human serum (Fig. 4).

The glutathione surfaces did not deposit detectable amounts of any of the antisera tested for. The thickness of the total adsorbed protein layer after plasma incubations and rinsings was less than 1 nm. This suggests that
5 GSH, when chemisorbed onto gold, presents a surface with advantageous properties for blood contact applications.

Another intriguing observation in Figures 4 and 5, was the large difference in the adsorbed amounts of plasma and serum protein onto the modified surfaces. This difference was particularly large for GSH. Control tests with
10 heparinized plasma in PBS buffer and citrated plasma in citrate buffer, showed no elevated depositions of organic material on GSH.

Thus, in summary it can especially be seen (Fig. 5a-
15 b) that pure gold (Au) and MPA bind large amounts of α -HMWK, indicating contact activation of coagulation. L-cysteine shows increased binding of α -IgG, α -ATh III and α -Lipoprotein. This combined with the results of Figure 4 (showing increased α -C3c binding onto serum incubated L-
20 cysteine surfaces) indicate that L-cysteine imparts some activation to the complement system but not the coagulation. On the contrary, glutathione does not activate any of these systems.

Finally, preliminary results from vital microscopy
25 experiments on rats confirm that GSH-surfaces have a pronounced low activation of the inflammatory system. Thus, it has been observed that platelets from body fluids were associated to the GSH-surfaces but were not activated during the test period.

30

35

Table 1. Physical surface characterization

	Au	Au+MPA	Au+L-cys	Au+GSH
Static water contact angle (degrees)	<20	14±3	5±3	7±2
Refractive index at λ=632.8 nm	(0.16±0.02) +i(5.63±0.02)	(0.18±0.02) +i(3.54±0.02)	(0.18±0.02) +i(3.54±0.02)	(0.19±0.04) +i(3.51±0.03)
Layer thickness (nm)	-	0.6 ± 0.2	0.6 ± 0.2	1.3 ± 0.2
Surface roughness (nm) (rms-value)	<6	<6	<6	<7

The errors given as maximum errors

Table 2. FT-IRAS characterization of MPA, L-cys and GSH on gold. Peak positions are given in wavenumbers (cm^{-1}).

MPA	L-cys	GSH	Assignment
1720	1714	1728	$\nu_{\text{C=O}}$
		1663	Amide I
	1647		$\delta_{\text{as}}\text{NH}_3^+$
		1549	Amide II
	1514		$\delta_{\text{s}}\text{NH}_3^+$
1408	1421	1408	$\nu_{\text{t}}\text{CO}_2^-$
	1396		
1329			
		1242	Amide III
1223			
	1144		
1053			
974			$\text{O-H}_{\text{opb}}(\text{facing})$

$\nu_{\text{s/as}}$: symmetric/asymmetric stretching mode

$\delta_{\text{s/as}}$: symmetric/asymmetric deformation mode

δ_{sc} : scissoring mode

opb : out-of-plane bend

CLAIMS

1. A substrate having at least a surface layer modified by an immobilized thiol compound, characterized in
5 that said surface layer is a noble metal or polymer layer modified by immobilized glutathione.

2. A substrate according to claim 1, characterized in that the noble metal is selected from the group consisting of gold, silver and platinum.

10 3. A substrate according to claim 2, characterized in that the noble metal is gold.

4. A substrate according to claim 1, characterized in that the polymer is selected from the group consisting of a polyethylene, polypropylene, polystyrene, polyurethane
15 or terephthalate polymer.

5. A substrate according to any one of the preceding claims, characterized in that said surface layer is a 50 - 500 nm thick layer on another substrate, preferably selected from the group consisting of silicon, glass, polymer
20 and metal.

6. A substrate according to claim 5, characterized in that said surface layer is 100-300 nm thick.

7. A substrate according to any one of claims 5 and 6, characterized in that said noble metal layer has been
25 sputter- or vapourdeposited on said substrate.

8. A substrate according to any one of the preceding claims, characterized in that the thickness of the immobilized glutathione layer is within the range of 5-25 Å, preferably 5-13 Å.

30 9. A substrate according to any one of the preceding claims, characterized in that said glutathione is present as a monolayer immobilized on said surface layer.

10. A substrate according to any one of the preceding claims, characterized in that the glutathione has been
35 immobilized on said surface layer by chemisorption from an aqueous solution thereof.

11. A substrate according to claim 10, characterized in that the concentration of said aqueous solution of glutathione is 0.1-10 mM.

12. A process for the preparation of a substrate
5 having at least a surface layer modified by an immobilized glutathione layer as defined in any one of claims 1-11, characterized by starting from a substrate having a noble metal or polymer surface or alternatively sputter-depositing on a substrate a noble metal surface and modifying
10 said surface layer with glutathione by chemisorption from an aqueous solution of said glutathione so as to immobilize the same thereupon.

13. A process according to claim 12, characterized by sonication of the chemisorbed glutathione layer, preferably
15 after rinsing thereof in water.

14. An article comprising a substrate as defined in any one of claims 1-11 or manufactured as defined in any one of claims 12-13, for use in medical or diagnostic treatment of the human or animal body, especially for
20 blood contact treatments thereof.

15. An article according to claim 14, characterized in that it is selected from the group consisting of implants, surgical instruments and sensors.

16. An article according to claim 14, characterized
25 in that it is intended for cell culture uses.

17. Use of a substrate as defined in any one of claims 1-11 or manufactured as defined in any one of claims 12-13 for the manufacture of an article to be used in blood contacting medical or diagnostic treatments or
30 surgical treatment of the human or animal body.

18. Use according to claim 17 for the manufacture of an implant or surgical instrument article.

1/6

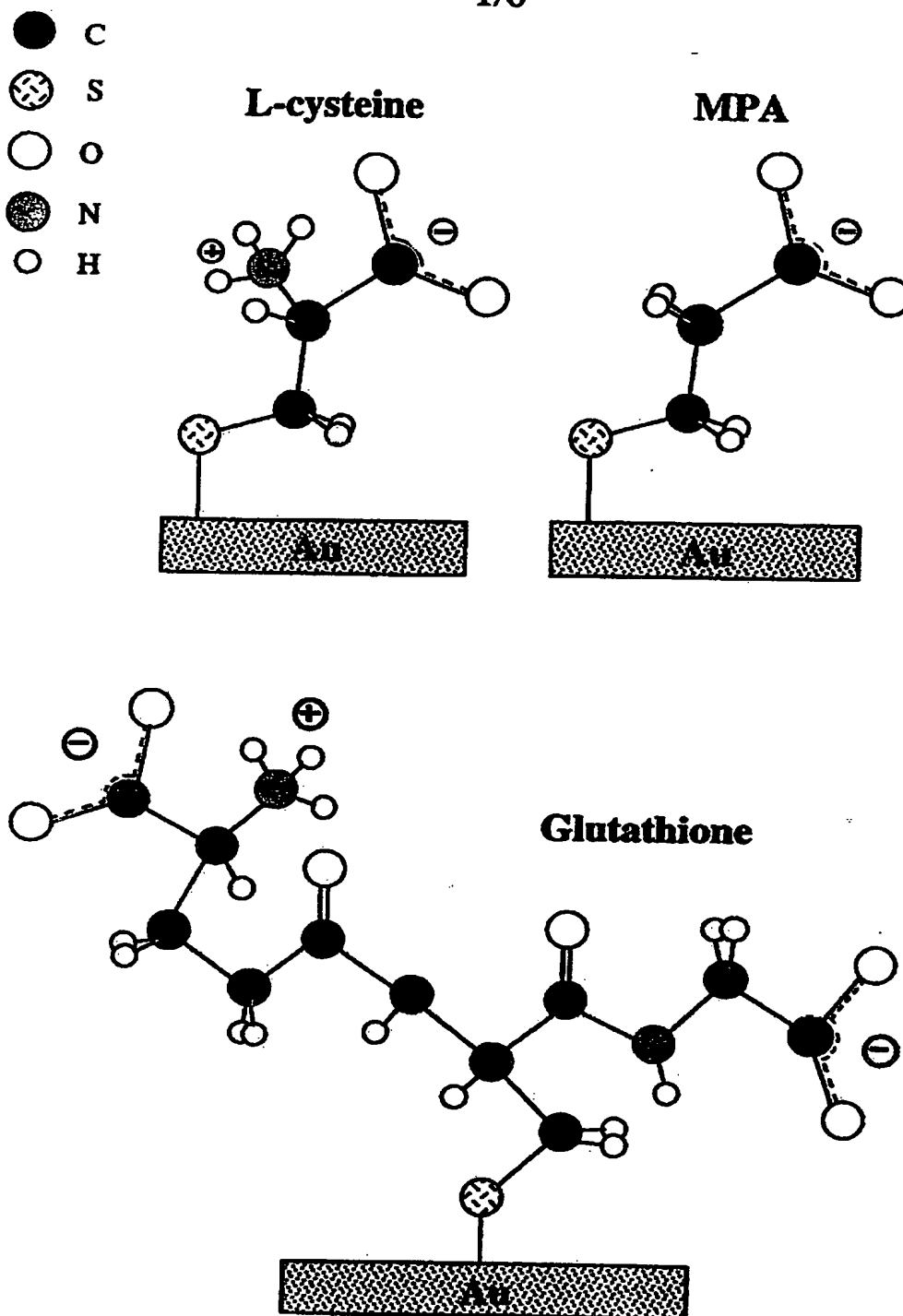


Figure 1.

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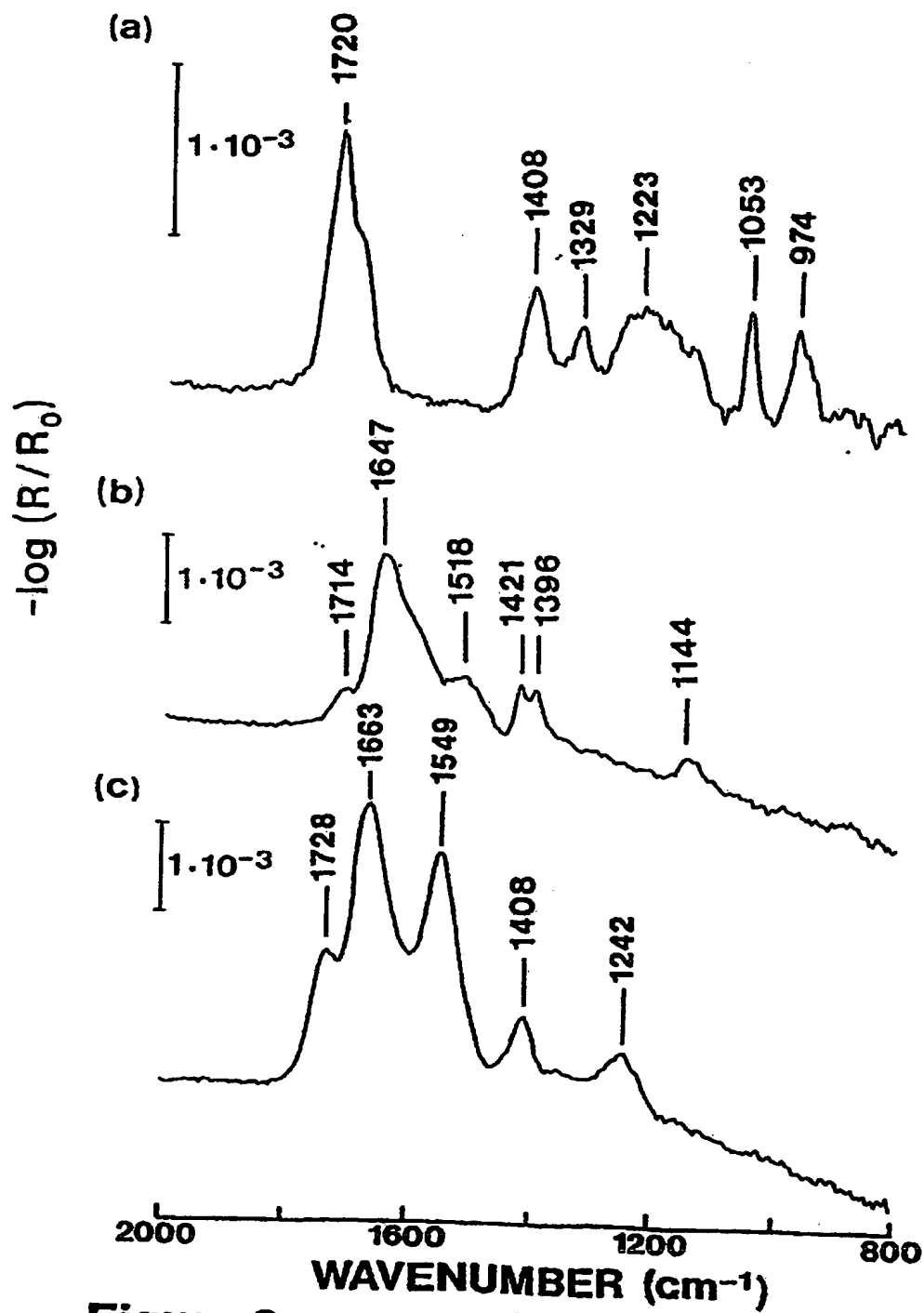


Figure 2

SUBSTITUTE SHEET

3/6

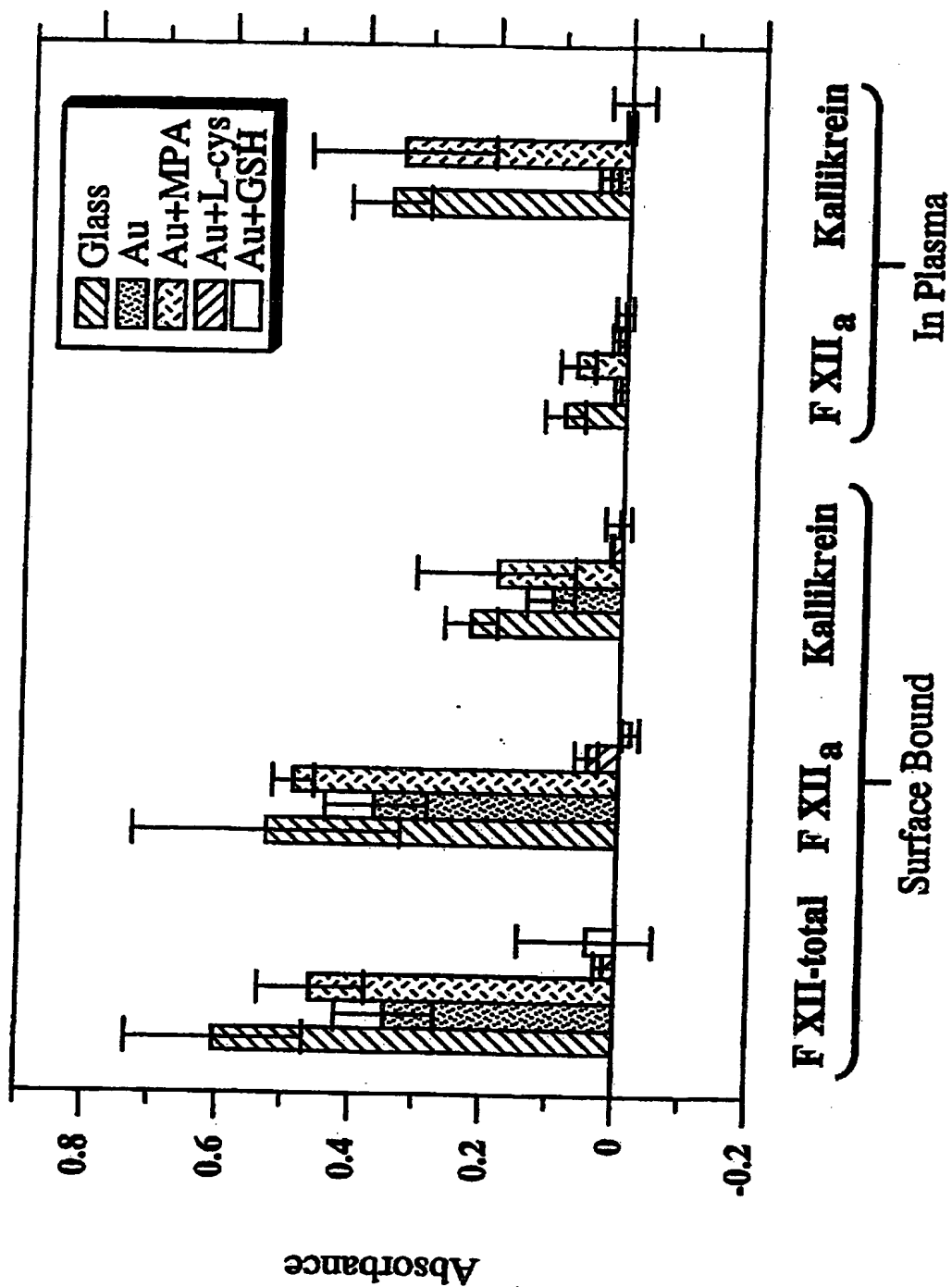


Figure 3.

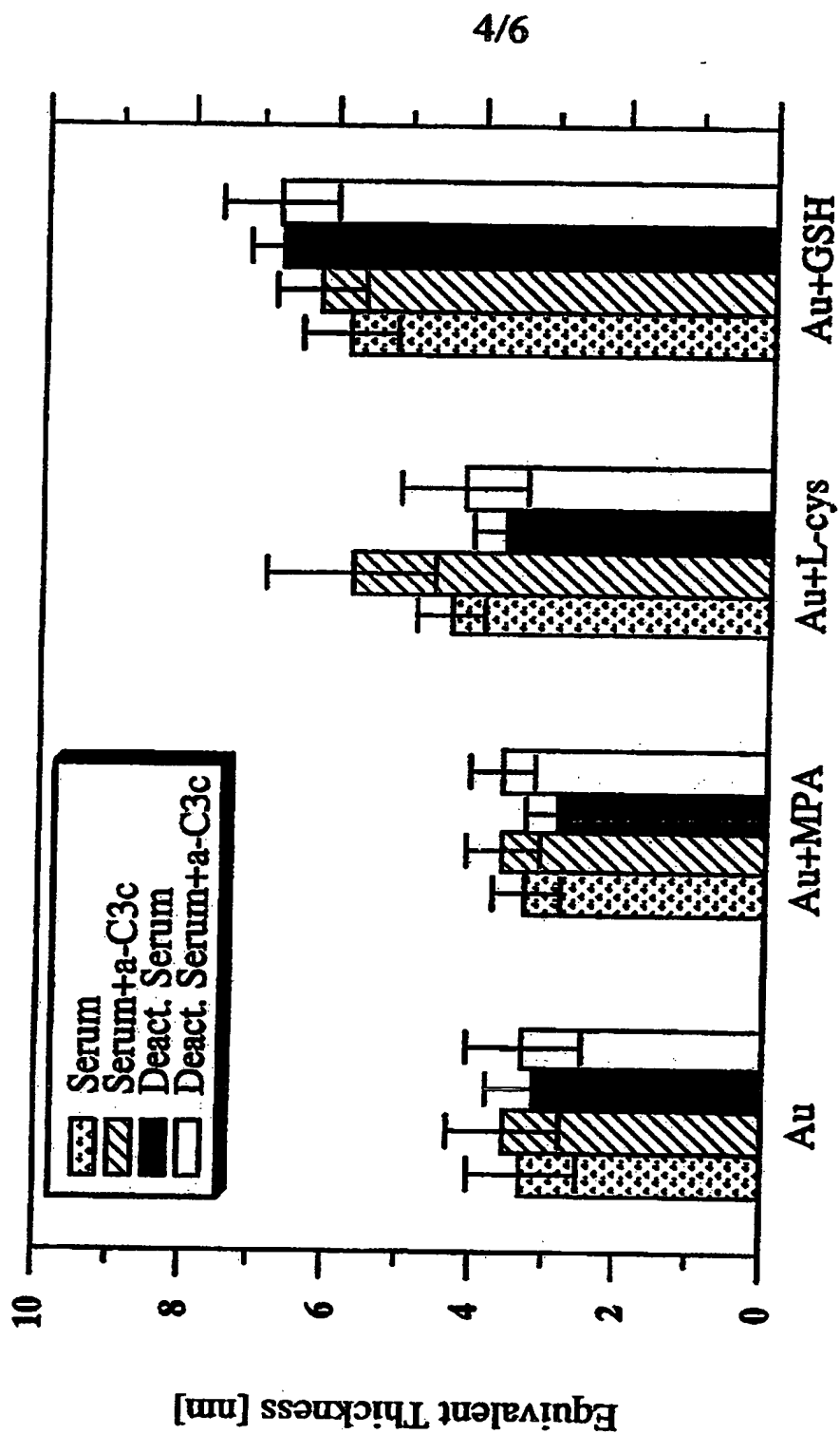


Figure 4.

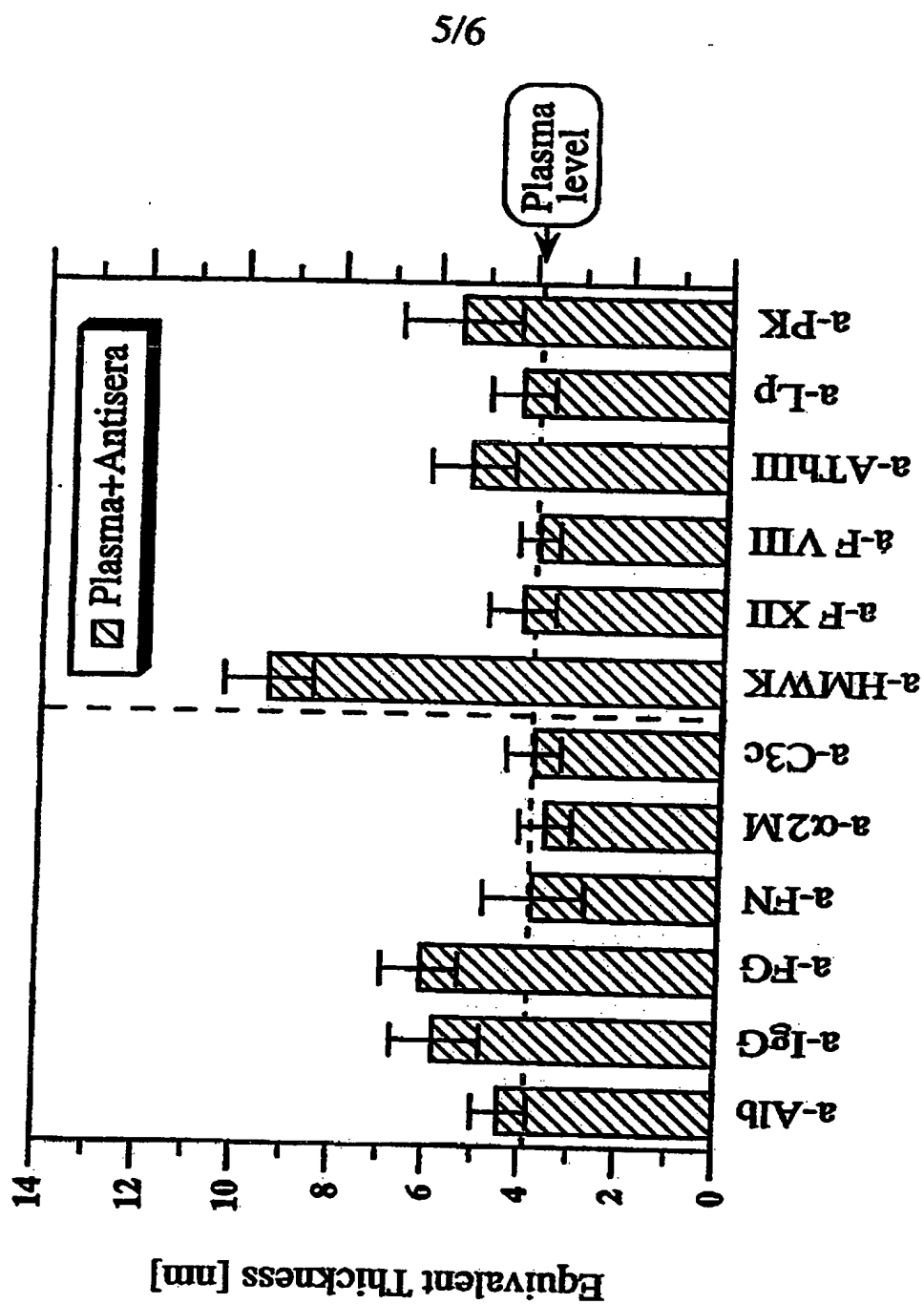


Figure 5a.

6/6

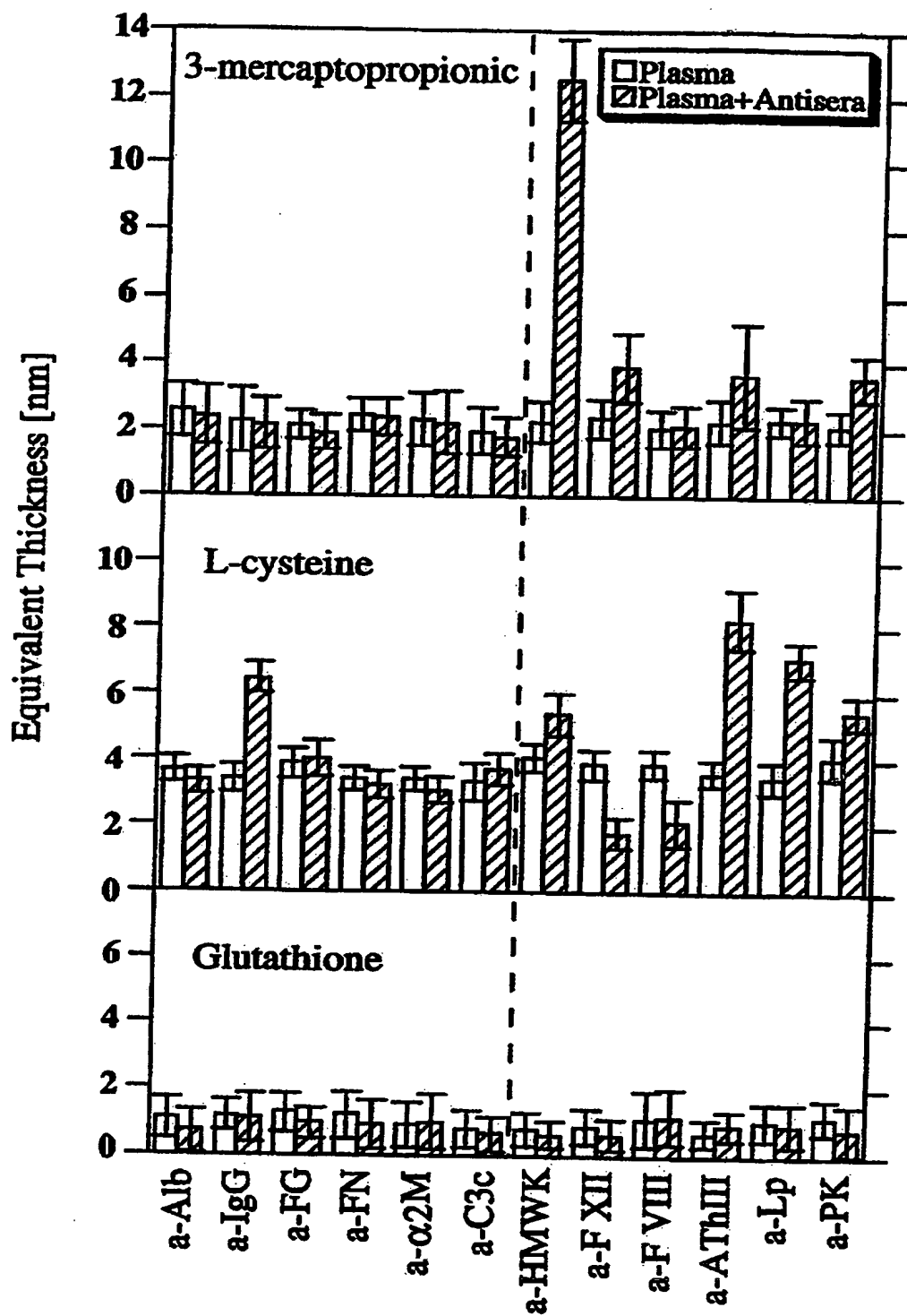


Figure 5b.

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 95/00825

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: G01N 33/553, A61L 33/00, G01N 33/545, A61L 27/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: G01N, A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

VIA DIALOG: BIOSIS, MEDLINE, WPI, SCISEARCH, VIA ORBIT: WPI, USPM, EDOC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0485874 A2 (F. HOFFMANN-LA ROCHE AG), 20 May 1992 (20.05.92) --	1-3,5-16
X	Dialog Information Services, file 55, Biosis, Dialog accession no. 9051642, Biosis accession no. 93036642, Batista-Viera F.: "A new method for Reversible Immobilization of Thiol Biomolecules Based on Solid-Phase Bound Thiol Sulfonate Groups", Appl Biochem Biotechnol 31 (2). 1991. 175-195 --	1,4-6,8-9, 14-18
A	WO 9322320 A1 (BIOCOMPATIBLES LIMITED), 11 November 1993 (11.11.93) -- -----	1-3,5-18



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 October 1995

Date of mailing of the international search report

06 -11- 1995

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Patrick Andersson
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 95/00825

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 95/00825

According to rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over prior art.

Such a link between all the subject of claims 1-18 would be immobilisation of glutathion. This *a priori* allegation however is not acceptable due to the state of the art as revealed in the attached search report, i.e Batista-Viera et al.. Accordingly, the following inventions were found:

Invention 1: claim 2-3,7 completely and claims 1,5-6 and 8-18 partially, a substrate, its use and preparation, having a surface layer of noble metal with glutathion immobilised on to it.

Invention 2: claim 4 completely and claims 1,5-6 and 8-18 partially, a substrate, its use and preparation, having a polymer surface layer with glutathion immobilised on to it.

In spite of the non-unity all the claims have been included in the search.

INTERNATIONAL SEARCH REPORT

02/10/95

International application No.

PCT/SE 95/00825

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0485874	20/05/92	CA-A- 2055117 JP-A- 4268455	15/05/92 24/09/92
WO-A1- 9322320	11/11/93	NONE	

**Section 2. Forms PTO/SB/08A and 08B (formerly Form PTO-1449)****IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants: Miyata, T.

Attorney Docket: 2605/102

Serial No: 10/009,877

Art Group Unit: ~~Not Assigned~~ 1251

Date Filed: November 13, 2001

Examiner Name: ~~Not Assigned~~ Hanley

Invention: BLOOD CARBONYL COMPOUND TRAPPING AGENT

**LIST OF PATENTS AND PUBLICATIONS FOR
APPLICANT'S SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT**

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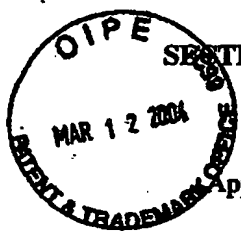
International Patents

Examiner Initials	Reference Number	Document Number	Issue Date	Inventor	Class/Subclass
SM/3	AM	WO 01/24790	2001 April 12	Miyata	A61K 31/155

Examiner Signature: [Signature]Date Considered: 7/2/04

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation *if not* in conformance and not considered. Include copy of this form with next communication to applicant.

02605/00102 212017.1



SECTION 2. FORMS PTO/SB/08A and 08B (formerly Form PTO-1449)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Toshio Miyata

Attorney Docket: 2605/102

Serial No: 10/009,877

Art Group Unit: 1614 1651

Date Filed: November 13, 2001

Examiner Name: Not Yet Assigned. Hanley, S.

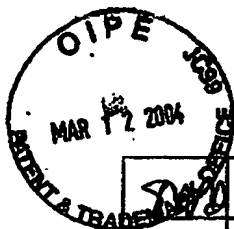
Invention: Blood Carbonyl Compound-Trapping Agent

LIST OF PATENTS AND PUBLICATIONS FOR
APPLICANT'S SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

U.S. PATENT DOCUMENTS					
Examiner Initials	Reference Number	Document Number	Issue Date	Inventor	Class/Subclass
SMA	DP	US5,128,360	07/07/1992	Cerami, et al.	514/400
	DQ	US5,827,820	10/27/1998	duMoulin, et al.	514/2
	DR	US5,855,882	01/05/1999	Li, et al.	424/94.61
	DS	US5,861,238	01/19/1999	Li, et al.	435/2
	DT	US5,891,341	04/06/1999	Li, et al.	210/646
	DU	US5,962,245	10/05/1999	Li, et al.	435/18

FOREIGN PATENT DOCUMENTS						
Exam. Initials	Ref. No.	Country Code	Doc. No.	Public. Date	Patentee or Applicant	Class/Subclass
SMA	DV	JP	5-105633, A and corresponding English translation.	4/27/93	Sato, T.	A61K 31/70
	DW	JP	4-187158, A and corresponding English abstract	7/3/92	Masuda, T., et al.	A61M 1/28
	DX	JP	8-131542, A and corresponding English translation	5/28/96	Izumi, G., et al.	A61M 1/14
	DY	JP	6-507822, A See Ref. DQ for corresponding US Application	9/8/94	duMoulin, A., et al.	A61M 1/28
	DZ	JP	63-19149, A (We Could Not Obtain any English Translation or a Concise English Explanation of this Document)	1/26/88	Suzuki, T, et al.	A61 J 1/00
SMA	EA	WO	93/19792 (Ref. DQ is a continuation of this application)	10/14/93	duMoulin A, et al.	A61M 1/28

OTHER DOCUMENTS			
Exam. Initials	Ref. No.	Author	Title of Article, Title of Journal, Volume Number, Page Numbers, Date
SMA	EB	Tanaka Y, et al.	Inhibitory Effect of Metformin on Formation of Advanced Glycation End Products, <i>Current Therapeutic Research</i> , Vol. 58, No. 10 (10/1997) pp. 693-697.



<input checked="" type="checkbox"/>	EC	Lo TWC, et al.	Binding and Modification of Proteins by Methylglyoxal Under Physiological Conditions, <i>J Biol Chem</i> , Vol. 269, No. 51 (12/23/1994): pp. 32299-32305
<input checked="" type="checkbox"/>	ED	Niquette, P., et al.	Backwashing First-Stage Sand-BAC Filters, <i>J Am Water Works Assoc</i> , Vol. 90, Issue 1 (January, 1998), pp 86-97
<input checked="" type="checkbox"/>	EF	Combet, S., et al.	Vascular Proliferation and Enhanced Expression of Endothelial Nitric Oxide Synthase in Human Peritoneum Exposed to Long-Term Peritoneal Dialysis, <i>J Am Soc Nephrol</i> , 11:717-728 (2000)
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<input checked="" type="checkbox"/>	EJ	Miyata, T., et al.	Accumulation of Carbonyls Accelerates the Formation of Pentosidine, an Advanced Glycation End Product: Carbonyl Stress in Uremia, <i>J. Am Soc Nephrol</i> , 9:2349-2356 (1998).
<input checked="" type="checkbox"/>	EK	Miyata, T., et al.	Autoxidation Products of Both Carbohydrates and Lipids are Increased in Uremic Plasma: Is there Oxidative Stress in Uremia?, <i>Kidney Intl</i> , Vol. 54 (1998), pp. 1290-1295.
<input checked="" type="checkbox"/>	EL	Miyata, T., et al.	Alterations in Nonenzymatic Biochemistry in Uremia: Origin and Significance of "Carbonyl Stress" in Long-Term Uremic Complications, <i>Kidney Intl</i> , Vol. 55 (1999) pp. 389-399.
<input checked="" type="checkbox"/>	EM	Nakayama, M., et al.	Immunohistochemical Detection of Advanced Glycosylation End-Products in the Peritoneum and its Possible Pathophysiological Role in CAPD, <i>Kidney Intl</i> , Vol. 51 (1997) pp. 182-186.
<input checked="" type="checkbox"/>	EN	Wilkie, MB, et al.	Polyglucose Solutions in CAPD, <i>Perit Dial Intl</i> , Vol. 17, (1997), pp. S47-S50.
<input checked="" type="checkbox"/>	EO	Yamada, K., et al.	Immunohistochemical Study of Human Advanced Glycosylation End-Products (AGE) in Chronic Renal Failure, <i>Clin Nephrol</i> , Vol. 42, No. 6 (1994) pp. 354-361.

Examiner Signature: _____

Date Considered: _____

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

**Section 2. Forms PTO/SB/08A and 08B (formerly Form PTO-1449)****IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants: Miyata, T.

Attorney Docket: 2605/102

Serial No: 10/009877

Art Group Unit: 1651

Date Filed: November 13, 2001

Examiner Name: Hanley

Invention: BLOOD CARBONYL COMPOUND TRAPPING AGENT

**LIST OF PATENTS AND PUBLICATIONS FOR
APPLICANT'S INFORMATION DISCLOSURE STATEMENT****United States Patents**

Examiner Initials	Reference Number	Document Number	Issue Date	Inventor	Class/Subclass
<u>SMH</u>	AA	3,284,531	Nov. 8, 1966	Shaw et al.	260/677
<u>SMH</u>	AB	3,793,187	Feb. 19, 1974	Marx et al.	208/289

International Patents

Examiner Initials	Reference Number	Document Number	Issue Date	Inventor	Class/Subclass
<u>SMH</u>	AC	WO 96/31537	Oct. 10, 1996	Li et al.	C07K 14/79
<u>SMH</u>	AD	WO 00/10606	Mar. 2, 00	Miyata	A61K 45/00

Other Documents

Examiner Initials	Reference Number	Author	Title of Article, Title of Journal, Volume Number, Page Numbers, Date
<u>SMH</u>	AE	Ungar et al.	"Inhibition of Binding of Aldehydes of Biogenic Amines in Tissues", Biochemical Pharmacology, Vol. 22, pp. 1905-1913, 1973



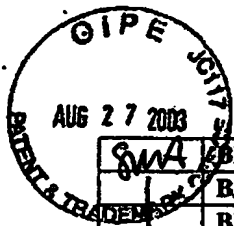
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- AL Miyata et al. "2-Isopropylidenehydrazono-4-oxo-thiazolidin-5-ylacetanilide (OPB-9195) treatment inhibits the development of intimal thickening after balloon injury of rat carotid artery: role of glycooxidation and lipoxidation reactions in vascular tissue damage", FEBS Letters, Vol. 445, pp. 202-206, 1999

Examiner Signature: *A. B. [Signature]*

Date Considered: 7/2/04

EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation *if not* in conformance and not considered. Include copy of this form with next communication to applicant.

02605/00102 189221.1



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SMW	BI	JP	10-158244, A	6/16/98	Iyobe, A., et al.	C07D213/65
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	BU	JP	62-249909, A *See Ref. No. DI for Corresponding EP Appln.	10/30/87	Nagaoka, Y., et al.	A61K7/00
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Applicants: Toshio Miyata

Attorney Docket: 2605/102

TECH CENTER 1600/2900

Serial No: 10/009,877

Art Group Unit: 4614-1651

Date Filed: November 13, 2001

Examiner Name: Not Yet Assigned

Hanley, S.

Invention: Blood Carbonyl Compound-Trapping Agent

LIST OF PATENTS AND PUBLICATIONS FOR
APPLICANT'S SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

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Examiner Initials	Reference Number	Document Number	Issue Date	Inventor	Class/Subclass
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